Obesity Modulates microRNA Expression in the Visceral Adipose of Humans and Mice

Honors Thesis Research

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By

Dheeraj Duggineni

The Ohio State University

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Project Advisor: Dr. Sanjay Rajagopalan, College of Medicine

ABSTRACT

Objective: Previous studies have indicated that dysregulation of miRNAs in visceral adipose tissue of mice and omental/subcutaneous adipose tissue of humans are linked to obesity and insulin resistance. In this study, we profile the differential miRNA expression of obese IR bariatric patients and propose mechanisms of miRNA transport that may play a role in systemic inflammation/insulin resistance.

Methods and Results: Whole visceral adipose tissue from lean and obese patients undergoing bariatric surgery was analyzed for miRNA expression. Four miRNAs (miR-223, miR-486, miR-15b, and miR-451) were upregulated in obese patients and found to be overexpressed in the stromal vascular fraction (SVF) of the visceral adipose tissue. The overexpression of miR-223 in the SVF was also confirmed in a mouse model. Expression of miR-223 was found to be upregulated in mouse liver tissue, but not in mouse muscle tissue, human blood monocytes, or human adipocytes. Isolation of human plasma exosomes revealed miR-223 was selectively overexpressed relative to the other three miRNAs.

Conclusions: In this paper, we profile previously unreported obesity-related miRNA upregulation in the stromal vascular fraction of human visceral adipose tissue. Our results provide evidence that miRNA dysregulation during chronic obesity may play a key role in regulation of inflammation and insulin resistance of not only adipose tissue but of other tissues as well. We also show preferential enrichment of miR in the plasma exosome compartment and introduce the possibility of miRNA transport from viscera to distal metabolic organs.

INTRODUCTION

Chronic obesity represents a growing worldwide problem and is considered the sixth most important risk factor contributing to overall burden of disease [1]. Impacts on life expectancy due to obesity have namely been attributed to development of cardiovascular diseases and type 2 diabetes. However, obesity also results in a wide variety of pathophysiological conditions including insulin resistance, glucose intolerance, systemic inflammation, and metabolic disorders [1,2,3].

Obesity-related visceral adipose tissue changes include a significant infiltration of inflammatory immune cells (T cells and macrophages), adipocyte hypertrophy, and increased adipocyte cell death. Infiltrating macrophages and T-cells secrete inflammatory cytokines and the resulting inflammatory effects within adipose tissue have been well documented [4,5,6,7]. Using mouse models, the role of inflammatory cells / visceral adipose inflammation has been shown to be etiologically relevant in diet-induced obesity (DIO) and insulin resistance (IR) [8,9]. Understanding the pathogenic changes that occur in the visceral adipose during DIO is essential to developing effective therapeutics targeting IR/T2DM.

MicroRNAs (miRNAs) are small, ~22 nucleotide, highly-conserved, non-coding strands of RNA thought to play an important role in post-transcriptional regulation of a variety of cellular processes in eukaryotic organisms [10]. By base-pairing with complimentary “seed” sites within target mRNAs, miRNAs can cause degradation of the mRNA strand or translational repression [11,12,13]. An estimated 30% of the human genome is thought to be regulated by miRNAs. Of particular interest for this study,

miRNAs have been shown to play critical roles in regulation of adipogenesis, lipid metabolism, and inflammatory response [14,15]. Thus dysregulation of miRNAs in the obese state may play a role in visceral adipose inflammation and tissue dysfunction, potentiating metabolic disease development and/or progression.

Previous studies have indicated that dysregulation of miRNAs in visceral adipose tissue of mice and omental/subcutaneous adipose tissue of humans are linked to obesity and insulin resistance [16,17]. In particular, one study analyzed miRNA modulation after DIO in mice and found 22 differentially-expressed miRNAs compared to lean counterparts [18]. Further studies have identified dysregulation of miRNAs in subcutaneous and visceral adipose tissue of non-obese hyperglycemic individuals when compared to non-obese normoglycemic individuals [19]. Evidence suggests that adipose tissue miRNA dysregulation is linked to obesity and insulin resistance, especially in mouse models.

In this study, we profile the differential miRNA expression of obese IR bariatric patients. Moreover, we show conservation of miRNA regulation in a mouse model by analyzing adipose, liver, and muscle tissue. We show preferential enrichment of miR in the plasma exosome compartment and introduce the possibility of miRNA transport from viscera to distal metabolic organs.

METHODS AND MATERIALS

Human Participants

Visceral adipose tissue was obtained from the greater omentum of patients undergoing hernia repairs or bariatric surgery. Patients were stratified based on BMI (lean, BMI<30; obese, BMI≥40). Fasting blood was drawn the morning of surgery by venipuncture and plasma isolated using centrifugation. The Office of Responsible Research Practices, Human Institutional Review Board (IRB) of the Ohio State University under OSU protocol #2008H0177, has approved this study and its procedures. Human informed consent was obtained.

Human Tissue Processing

A portion of whole adipose sample was digested using collagenase and separated by centrifugation into stromal vascular fraction (SVF) and adipocyte/fat fraction (FF). The SVF, containing macrophages, monocytes, T-cells, B-cells, and endothelial progenitor cells—were lysed in TRIzol® reagent (Life Technologies) and frozen at -80° C until needed. The adipocyte fraction (FF) was frozen separately at -80° C.

Mouse Tissue

Previously banked mouse muscle, liver, and adipose tissue from ob/ob knockout mice (a genetic model of spontaneous obesity) and wild-type (WT) C57BL/6 controls were analyzed. The samples were from obesity experiments previously performed in the lab.

RNA Isolation

Prior to RNA isolation, tissue was homogenized in one milliliter TRIzol® reagent before lipids were removed via centrifugation. To extract RNA, chloroform (200 µl) was added to samples containing TRIzol. Centrifugation (12,000 x g, 15 min, 4° C) separated aqueous and organic layers. RNA was precipitated from the aqueous layer using isopropyl alcohol. Linear acrylamide was used to increase RNA yield. The RNA pellet was washed in ethanol before air drying. RNA was dissolved in RNase free water and quantified using Nanodrop microvolume spectrophotometer (Thermo Scientific). Purity of samples was determined by 260/280 and 260/230 ratios which measure DNA and protein contamination, respectively. RNA was stored at -80°.

Exiqon miRNA Analysis

1 µg of RNA from human whole visceral adipose was hybridized to an Exiqon miRCURY™ LNA Array 5th generation (product number 208300-A (208301-A / 208302-A, slide batch 33011) using miRBase 15.0 + miRPlus. Analysis of the scanned slides showed that the labeling was successful as all capture probes for the control spike-in oligo nucleotides produced signals in the expected range. The quantified signals (background corrected) were normalized using the global Lowess (LOcally WEighted Scatterplot Smoothing) regression algorithm. In the expression matrix, all capture probes with both Hy3 (sample) and Hy5 (common reference pool) signals lower than 1.5x of the median signal intensity of the given slide were excluded from analysis. Only log2(Hy3/Hy5) ratios which passed the filtering criteria on variation across samples were used. Student's t-test was performed between lean and obese patient groups. The

miRNAs with mean expression level change above 1.3-fold, and p value < 0.05 were further selected to check their correlation with patient phenotypic traits (BMI, CRP level, fasting insulin level, and HOMA index).

RT PCR for miRNA-Specific cDNA

MicroRNA specific cDNA was generated via the ABI TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). Briefly, 1 µl of 10X Reverse Transcriptase buffer, 0.75 µl of 10mM DNTPs, 0.5 µl of Reverse Transcriptase enzyme, 0.09 µl of RNase inhibitor, and 1.5 µl of microRNA specific primer were used to create a 3.84 µl RT master mix per sample. Then, 6.16 µl of RNA sample at 1 ng/ µl was added to the master mix to create a 10 µl reaction per sample. Samples were loaded into a thermocycler and reverse transcription reaction as described by the manufacturer was performed. Resulting microRNA cDNA samples were kept frozen at -80° C until needed.

qPCR Assay for miRNA cDNA

Quantitative PCR via ABI Taqman Probe Assay (Applied Biosystems) was performed to measure expression of target microRNAs in the tissue samples relative to a reference snRNA (RNU44). ABI Taqman Probe master mix (5.0 µl per reaction) mixed with 20X microRNA specific probe (0.5 µl per reaction) was used alongside 4.5 µl of microRNA specific cDNA to create a 10 µl reaction. Each sample was tested in duplicate. Expression of microRNA was quantified via fold change relative to RNU44.

RT PCR for Total cDNA

Total cDNA was generated from each RNA sample in order to test for gene expression. Briefly, a 5.0 µl master mix reaction composed of 1.0 µl of 10X RT Buffer, 1.0 µl of 10mM DNTPs, 1.0 µl of 10X ABI Random Primers (Applied Biosystems), 0.5 µl of Reverse Transcriptase enzyme, and 1.5 µl of RNase free H_20 was used per sample. Then, 350 ng of total RNA in a total volume of 5.0 µl H_20 was added to the master mix to create a 10 µl reaction per sample. Samples were loaded into a thermocycler and reverse transcription reaction was performed as per the manufacturers directions. Resulting total cDNA samples were kept frozen at -80° C until needed.

qPCR for Gene Expression

Quantitative PCR via SYBR Green Assay (Integrated Technologies) was performed to measure relative gene expression in tissue samples. Reactions consisting of 5.0 µl of SYBR Green master mix, 0.1 µl each of Forward and Reverse Primer, and 5.0 µl of total cDNA from samples were performed in duplicate per sample. Gene expression is expressed as fold change relative to *B-actin* housekeeping gene.

Isolation of Plasma Exosomes

Plasma was isolated from freshly collected EDTA-anti-coagulated blood. Differential centrifugation (16,500xg, 20”; 0.22 um filtration; 120,000xg, 180”) yielded secreted microvesicles (exosomes 50-200nm in size) and lipoproteins. RNA was isolated from exosomal pellet via Exiqon miRCURY™ RNA isolation kit. miR-specific cDNA was synthesized using equal volumes of RNA. miRNA expression was quantified by miRNA qPCR analysis as previously described.

RESULTS

Exiqon Array Profiling of Patients

Exiqon array data was analyzed by a consulting bioinformatics specialist who determined that 12 microRNAs passed the threshold of mean fold change > 1.3 and p-value < 0.05 between the lean and obese groups with three microRNAs being down-regulated (miR-299-3p, miRPlus-E1067, and miRPlus-E1076) and nine up-regulated (miR-202, -15b, -451, -24-2*, 1184, -187*, -486-5p, -10b, and -223). Hierarchical bi-clustering organized patients independent of group, re-arranging both axes by the correlation of expression profiling patterns (Figure 1). Hierarchical bi-clustering separated lean (blue) and obese (black) samples to groups relatively faithfully, with the exception of patient V28 and V14. Biomorphic characteristics of the sample population used in this experiment are shown in Table 1. Pearson correlation calculations were performed between data obtained from adipose tissue microRNA expression profiling and BMI, fasting insulin, CRP, and HOMA-IR. Of particular interest for this study were miR-223, mir-451, mir-15b, and mir-486, all of which correlated highly to the analyzed biomarkers (Table 2).

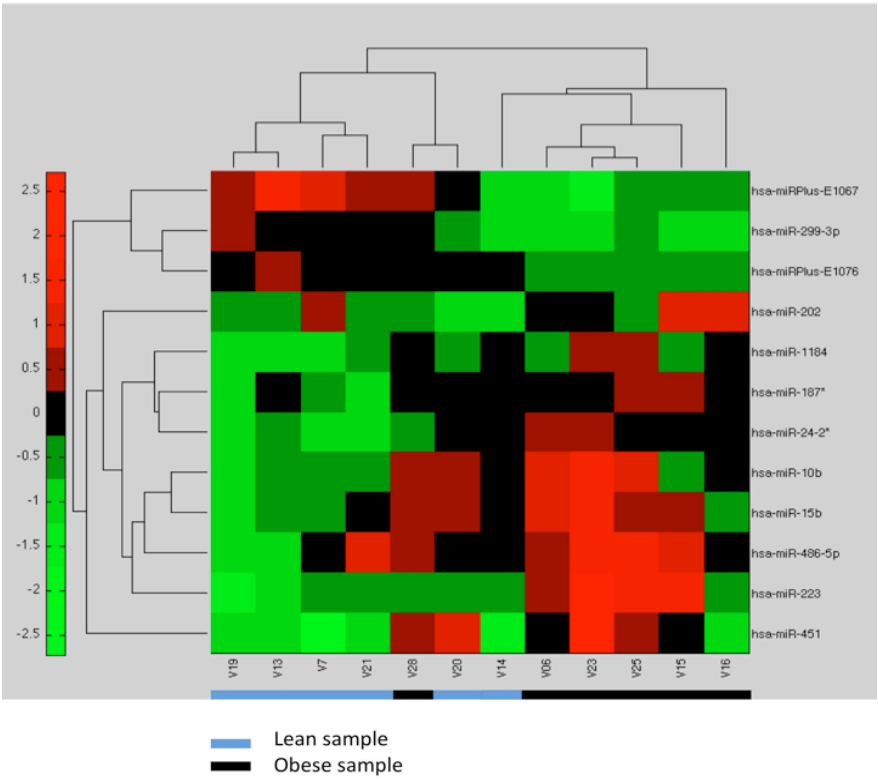

Figure 1. Heirarchial biclustering of heatmap data of 12 significantly changed miRNAs automatically clusters lean and obese groups. This is indicative of correlation between miRNAs and obesity.

Table 1. Biomorphic comparison of patients for Exiqon analysis

	Lean	Obese
Age	41.7±6	47.6±5
Gender	4F; 2M	5F; 1M
BMI	27±0.6	50±3*
Glucose	90±10	143±20*
Insulin	3.4±1.2	19.3±3.1*
HOMA-IR	0.77±0.3	6.7±1.3*
QUICKI	0.43±0.02	0.29±0.01*
HemA1c	5.6±0.1	6.6±1
TG	123±31	144±32
CRP	2.4±0.8	8.3±3.4
TC	171±9	168±14
HDL	44±4.3	41±7.5

LDL	102±11	98±10
TZD	0	3

Table 2. Pearson correlation coefficients of significant correlations between miRNA expression and the patient phenotypical traits.

miR	BMI	Fasting Insulin	HOMA	CRP
miR-223	0.80 p=0.002	0.66 p=0.027		0.63 p=0.038
miR-486-5p	0.74 p=0.006	0.72 p=0.013	0.70 p=0.016	0.68 p=0.021
miR-451	0.74 p=0.006	0.61 p=0.047	0.61 p=0.044	0.61 p=0.046
miR-15b	0.74 p=0.006	0.67 p=0.023	0.66 p=0.028	0.62 p=0.040

Determining the source of obesity-related miR-223 expression

To determine which fraction of adipose tissue is responsible for increases of miR-223 expression, lean (n=7) and obese (n=7) stromal vascular fraction (SVF) and fat fraction (FF) samples from the same patients were analyzed via qPCR for expression of miR-223. A significant increase in miR-223 expression was observed in the SVF obese samples (4.9-fold increase, p-value < 0.05) relative to SVF lean samples (Figure 2A). However, no difference was observed between the lean and obese groups in the FF samples relative to each other or relative to SVF lean samples. This indicates that SVF fraction is responsible for the observed increases in miR-223. Expression of miR-223 in monocytes isolated from patient blood samples were also analyzed (Figure 2B). No difference was found in these samples.

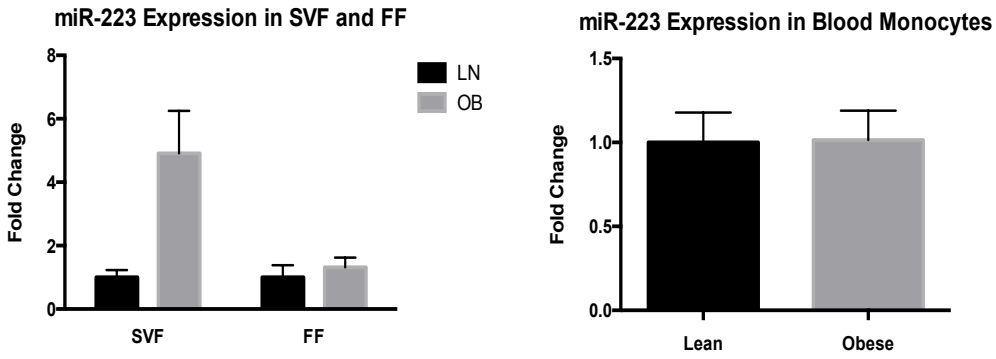

Figure 2. Quantitative PCR was used to measure miR-223 expression in SVF and FF of both lean and obese patients (A). Fold change is expressed relative to levels of SVF LN. The same patients used for SVF LN and OB were also used in the FF LN and OB. n=7 for each LN and OB group. Expression of miR-223 was also analyzed in blood monocytes. No difference was found.

qPCR Verification of Exiqon Data

Quantitative PCR analysis of RNA isolated from the SVF of lean and obese patients indicates there is a significant (p-value < 0.05) upregulation of mir223 (3.3-fold increase), mir15b (2.4-fold increase), mir451 (2.4-fold increase), and mir486 (2.3-fold increase) in obese patients when compared to their lean counterparts (Figure 3). These results were confirmed in replicate experiments. RNU44 was used as the housekeeping miRNA in these experiments. Biomorphic characteristics of the sample population used in this experiment are shown in Table 3.

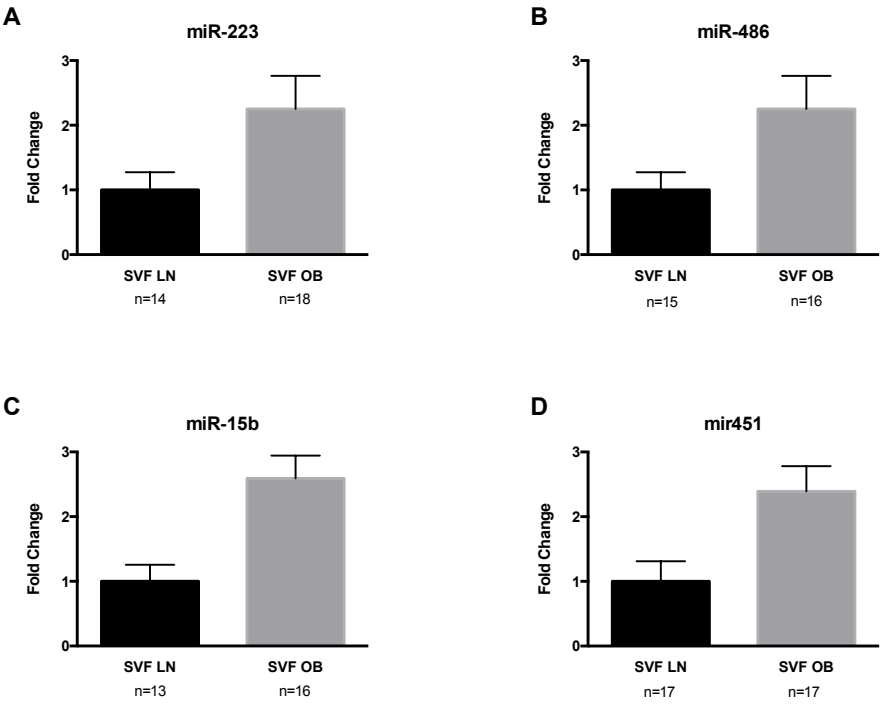

Figure 3. Relative miR-223 (A), miR-486 (B), miR-15b (C), and miR-451 (D) expression in SVF of lean and obese patients was measured via ABI TaqMan Probe qPCR kit. RNU44 was used as the houskeeping gene.

Table 1. Biomorphic comparison of patients used for qPCR analysis

	Lean	Obese
Age	47.1±15	44.3±10
Gender	9F;10M	10F; 9M
BMI	25.1±2	52.1±8*
Glucose	88±8	132±36*
Insulin	4.5±2.8	17.1±12*
HOMA-IR	1.0±0.6	5.8±5*
QUICKI	0.4±0.04	0.3±0.05*
HemA1c	5.4±0.4	6.1±1
TG	86±74	124±56
CRP	2.0±2.8	8.2±5.4
TC	173±54	163±29
HDL	52±16	37±7.5*
LDL	103±47	101±28

TZD	0	5

miR-451 is Highly Expressed in SVF

Quantitative PCR was used to analyze expression all four microRNAs of interest (mir223, mir451, mir15b, mir486) relative to each other in SVF samples (n=3). This was achieved by running reactions on one plate and normalizing fold difference relative to the lowest expressed microRNA, as identified by CT value. These reactions were run in duplicate. Mir-451 is expressed over 40 times higher than the other microRNAs of interest (Figure 4). This dramatic difference in expression suggests a more important physiological role for mir451.

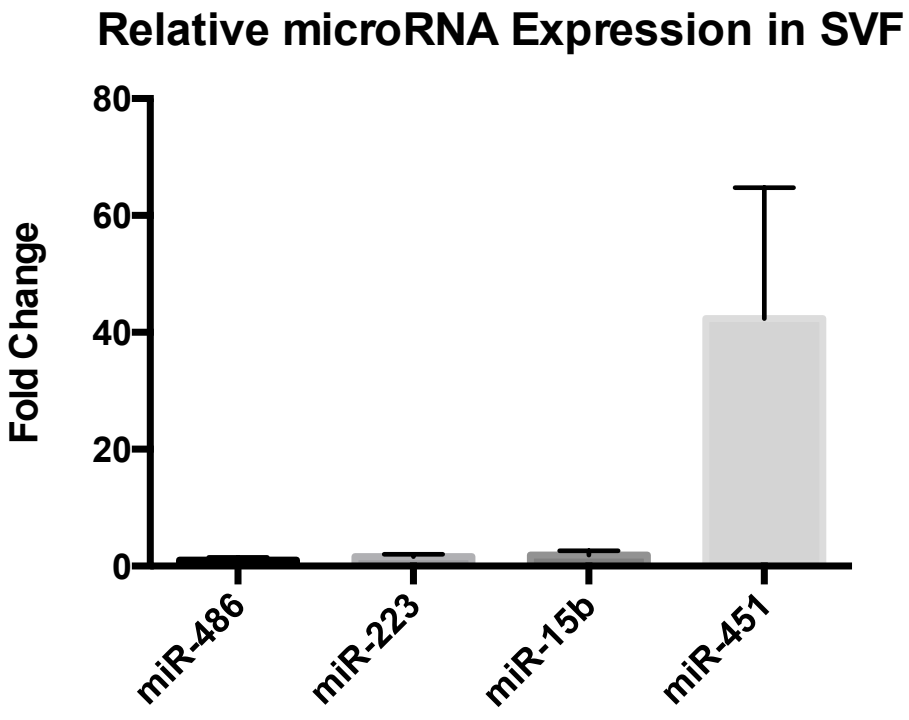

Figure 4. Quantative PCR was used to assess relative expression levels of microRNAs of interest in the SVF. Fold change is expressed relative to miR-486 expression levels (i.e. miR-486 fold change is 1). For each microRNA, n=3. Same patient samples were used for each microRNA

miR-486 Gene Target Expression unchanged in human samples

Quantitative PCR via SYBR Green was performed on SVF LN and OB samples to test for expression of SIRT-1, a putative gene target of miR-486 and an important glucose mediated cell cycle regulator [19]. No change was observed in gene expression between lean and obese patients (Figure 5). Thus any observed changes in SIRT-1 protein expression or SIRT-1 activity may be due to post-transcriptional regulation as is the case for most miRNA regulated genes.

Mouse Model Corroborates miR-223 Differential Expression

RNA isolated from ob/ob and WT mouse whole visceral adipose tissue was analyzed for miR-223 expression. There was a 2-fold increase in miR-223 expression in the ob/ob mouse adipose tissue when compared to wild-type counterparts. Furthermore, analysis of liver tissue found a 3-fold increase in miR-223 expression in ob/ob mice. These data suggest that the liver may play a vital role in miR-223-modulated insulin resistance. Analysis of muscle tissue from ob/ob and WT mice found no difference in miR-223 expression (Figure 6).

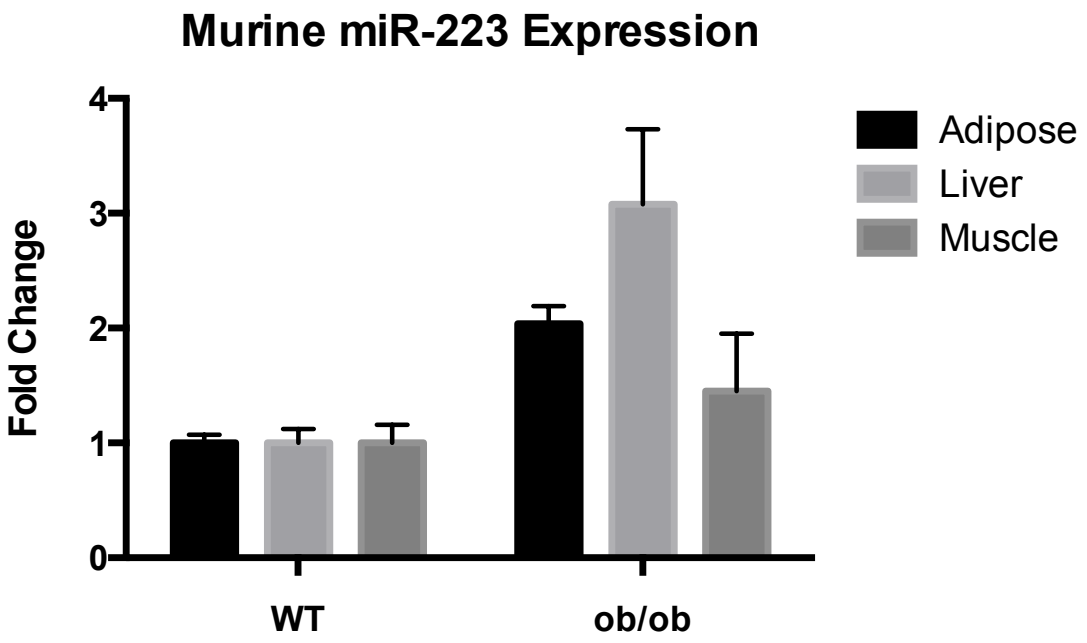

Figure 6. qPCR analysis reveals significant increase in miR-223 expression in adipose and liver tissue of ob/ob mice when compared to the WT control. n=8/group

Plasma Exosomes Derived from Obese humans are preferentially enriched in miR-223

Exosomal RNA isolated from plasma was analyzed for miRNAs of interest via ABI TaqMan Probe qPCR. All miRNAs of interest (miR-223, miR-451, miR-15b, miR-486) were expressed in the exosomes (Figure 7A). However, only miR-223 was differentially expressed between lean and obese samples. There was approximately a 9-fold (p-value < 0.05) increase of miR-223 expression in obese samples compared to lean samples (Figure 7B).

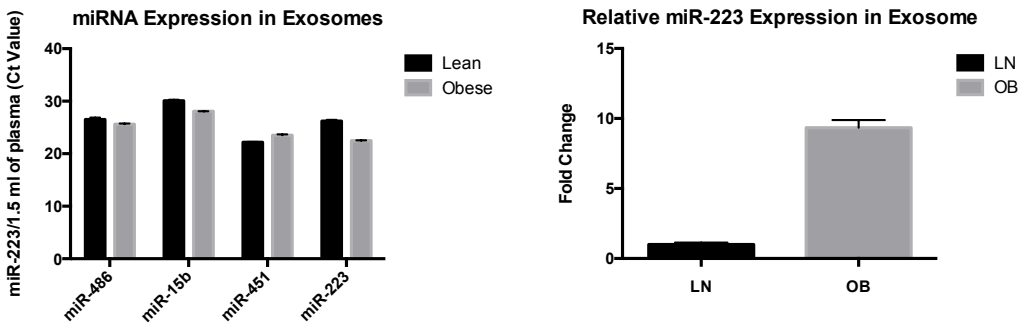

Figure 7. miR-486, miR-15b, miR-451, and miR-223 are all expressed in exosomes isolated from human plasma (A). miR-223 expression is increased 9-fold in the exosomes.

DISCUSSION

In this study we profile miRNAs in human adipose tissue that are differentially expressed in lean and obese patients. We then used a mouse model to confirm that this expression is conserved. Our results provide evidence that miRNA dysregulation during chronic obesity may play a key role in regulation of inflammation and insulin resistance of not only adipose tissue but of other tissues as well.

Exiqon data showed significantly increased expression of miR-223, miR-486, miR-451, and miR-15b in in the visceral adipose tissue of obese patients compared to their lean counterparts. Pearson correlation curves indicated that all four miRNAs have strong correlations with BMI, fasting insulin, and C-reactive protein and all but miR-223 have a strong correlation to HOMA-IR. These correlations suggest that expression of the miRNAs is correlated with obesity (as measured by BMI), insulin resistance (as measure by fasting insulin and HOMA-IR), and inflammation (as measure by C-reactive protein). Because of the correlations of these four miRNAs to relevant biomarkers, we pursued further analysis to characterize their adipose expression.

We determined that miR-223 was differentially expressed in the SVF of lean and obese patients but not in the FF or the blood monocytes. SVF is primarily composed of a mixed population of monocytes, macrophages, T-cells, and pre-adipocytes. Thus, the data suggests that this population of cells is solely responsible for the upregulation of miR-223 expression in the whole adipose rather than adipocytes which are primarily found in the FF. Based on previous studies, these results were expected. Recent studies have implicated miR-223 as critical regulator of macrophage activation. Putative

gene targets of miR-223 suggest that this miRNA plays a central role in regulation of ubiquitination/protein degradation (via *FBXw7*) and insulin signaling (via *IRS-1*) [19] miR-223 has also been shown to be highly expressed in myeloid cell populations [20].

Because miR-223 was highly expressed in SVF as opposed to FF, and because the other miRNAs of interest have previously been found in cells that comprise the SVF, we tested the SVF of a larger population of human samples for miRNA expression. Our results corroborated the Exiqon differential expression of miRNAs. Comparing relative expression levels of the miRNAs in the SVF found that miR-451 was much more highly expressed in the SVF than any other miRNA tested. This suggests that miR-451 may have a key pathophysiological role in adipose dysfunction. Evidence from previous studies indicate that miR-451 upregulation is induced by inflammatory cytokines such as IL-6 and type 1 Interferons [21]. Furthermore, miR-451 overexpression in gliomas have resulted in a downstream reduction of PI3K/AKT signaling [22]. This pathway is central to cell growth, apoptosis, and proliferation. Thus the high miR-451 expression found in the SVF could potentially be related to key role that miR-451 plays in overall cell function. Moreover, increases in miR-451, as is the case with the obese group, could result in apoptosis—not only potentiating inflammation of the visceral adipose tissue but also significantly contributing to adipose dysfunction.

Our mouse model studies not only indicate that obesity related miR-223 expression increase is a conserved finding in adipose tissue but that miR-223 expression is also increased in other tissue. It is important to note that increased miR-223 expression in the liver as a result of chronic obesity has not been previously described. The liver is a key organ in glucose metabolism and is rich in innate immune

cells. miR-223 dysregulation within this organ could result in changes in pathways relevant to DIO-IR.

Our study reveals that miR-223 is expressed in both adipose and liver tissue and previous studies have shown that miR-223 is expressed in mouse macrophages (both bone marrow derived and cell lines) as well as human hepatic cell lines. However, obesity induced differential miR-223 expression seems to be localized to specific tissues and cannot be found in circulating blood monocytes. Thus we proposed a novel mechanism of miR-223 obesity related modulation of insulin sensitivity via a visceral adipose tissue derived, miR-223 rich, exosome delivery to distant tissues. Our data shows a 9-fold increase in the expression of miR-223 in plasma derived exosomes thus providing preliminary evidence to support such a hypothesis. Exosomal delivery of miRNAs in general is not a novel mechanism. miRNA containing exosomes have been shown to exert paracrine-like effects in cardiovascular disease and cancer [23]. Macrophages have been known to regularly endocytose circulating exosomes and exosomal communication has been well characterized in immune systems [24,25].

Exosomal transport of miR-223 derived from the visceral adipose could provide a direct link between localized and systemic inflammation/insulin resistance. The challenge in diabetes and obesity has always been to find mechanistic links between local and systemic regulation. Obesity in general is pathologically localized to deposits of adipose tissue. Resulting adipose dysfunction is also localized. However, if miR-223 plays a central role in potentiating adipose dysfunction, then transport of miR-223 out of the adipose via exosomes provides a mechanistically sound link between local and systemic. Furthermore, miR-223 was found to be increased in the SVF. These cell

population have direct access to the blood and thus transport of miR-223 by secreted exosomes can prove to be truly systemic in scope. The challenge now will be to provide more evidence of visceral adipose tissue derived miR-223 transport by increasing sample size and characterizing destinations of the miRNA. Also, modulation of target genes needs to be analyzed to provide support to the idea that localized dysfunction can lead to systemic problems. Although the relative expression of the other miRNAs were not changed between lean and obese patients, this does not discount the potential for these miRNAs to play roles in adipose dysfunction or systemic problems.

Although this study provides a compelling profile of miRNA modulation exhibited during chronic obesity, these data can only provide preliminary/hypothetical framework of mechanistic effects of miRNA dysregulation. Modulation of putative gene targets of these miRNAs as predicted by DIANA program has yet to confirmed. To this end, 3' UTR luciferase assays need to be conducted for all miRNAs and target genes. Of particular interest and relevance is the canonical mTOR pathway that is involved in cell apoptosis, lipid metabolism, inflammation, and glucose metabolism. Furthermore, profiling of FF for all miRNAs can provide evidence to corroborate the data the suggests that SVF plays a much more significant role in the potentiation of adipose dysfunction and insulin resistance than FF.

This study provides significant evidence for the multidimensional/multisystem role of miRNAs in chronic obesity. Current trends are shifting from viewing diseases as localized problems to being more systemically relevant with a centrally regulating mechanism. Specifically, obesity by itself is not a burdensome disease. However, development of insulin resistance, inflammatory diseases, type 2 diabetes, and

cardiovascular diseases as a result of chronic obesity does create a significant burden. Finding the potential mechanisms of regulation of these systemic issues stemming from obesity—as we believe exosomal miR-223 transport may exhibit—can provide targets for drugs that provide a multisystem benefits. Furthermore, our mRNA profiling of chronically obese patients can provide other targets of interest for treating insulin resistance and preventing the development of type 2 diabetes.

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